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Phylogenetic species recognition and hybridisation in *Lasiodiplodia*: A case study on species from baobabs

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ABSTRACT

Lasiodiplodia species (*Botryosphaeriaceae*, *Ascomycota*) infect a wide range of typically woody plants on which they are associated with many different disease symptoms. In this study, we determined the identity of *Lasiodiplodia* isolates obtained from baobab (*Adansonia* species) trees in Africa and reviewed the molecular markers used to describe *Lasiodiplodia* species. Publicly available and newly produced sequence data for some of the type strains of *Lasiodiplodia* species showed incongruence amongst phylogenies of five nuclear loci. We conclude that several of the previously described *Lasiodiplodia* species are hybrids of other species. Isolates from baobab trees in Africa included nine species of *Lasiodiplodia* and two hybrid species. Inoculation trials with the most common *Lasiodiplodia* species collected from these trees produced significant lesions on young baobab trees. There was also variation in aggressiveness amongst isolates from the same species. The apparently widespread tendency of *Lasiodiplodia* species to hybridise demands that phylogenies from multiple loci (more than two and preferably four or more) are compared for congruence prior to new species being described. This will avoid hybrids being incorrectly described as new taxa, as has clearly occurred in the past.

Introduction

Species represent the basic units of taxonomy. However, decisions on how to define species boundaries, especially in fungi, are often problematic. Three species concepts are most commonly applied in fungal taxonomy, namely the Morphological (MSR), Biological (BSR) and Phylogenetic Species Recognition (PSR) concepts (Taylor *et al.* 2000) and all three present some challenges. Historically, fungal taxonomy has relied on the MSR concept, where species were described only when they could be distinguished based on distinct morphological characteristics (Taylor *et al.* 2000). The advent of DNA sequencing

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and an ability to apply phylogenetic inference has shown clearly that MSR has substantially underestimated the global fungal diversity (Crous *et al.* 2006; Schoch *et al.* 2014).

The BSR concept postulates that individuals of different species should be reproductively isolated (Taylor *et al.* 2000). However, there are growing numbers of examples where different species of fungi are able to cross and effectively reproduce to form hybrids. For example, a viable interspecies hybrid of *Fusarium circinatum* and *Fusarium subglutinans* has been produced under laboratory conditions (De Vos *et al.* 2011). Other examples include the hybrid poplar rust *Melampsora ×columbiana*, which is a natural hybrid of *Melampsora medusae* and *Melampsora occidentalis* (Newcombe *et al.* 2000), and the hybrids between the white pine blister rust *Cronartium ribicola* and *Cronartium comandrae* (Joly *et al.* 2006). An additional problem with the BSR concept is the fact that many fungi are known only in their asexual states and it is not possible to determine whether they are able to reproduce sexually.

The PSR concept, and more specifically the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) concept, is increasingly widely used to delineate species of fungi. This approach relies on determining the concordance between multiple gene genealogies and delimiting species where the branches of multiple trees display congruence (Taylor *et al.* 2000). The GCPSR ensures that species are not described based on small differences arising from within taxon variation.

The PSR has been widely applied during the last decade to describe cryptic species that could not be identified using the MSR. One example where a number of cryptic species have been described is in *Lasiodiplodia*, a common genus in the *Botryosphaeriaceae* (Phillips *et al.* 2013). The type species of this genus, *Lasiodiplodia theobromae,* has been reported from more than 500 plant species (Punithalingam 1976). This was, however, before the advent of DNA sequence-based identification (Pavlic *et al.* 2004; Slippers *et al.* 2004; Alves *et al.* 2008; Pavlic *et al.* 2009; Phillips *et al.* 2013). For many years *L. theobromae* was the only species in *Lasiodiplodia*, but 28 additional species have been described since 2004, based on both DNA sequence data and morphological characteristics (Pavlic *et al.* 2004; Burgess *et al.* 2006; Damm *et al.* 2007; Alves *et al.* 2008; Pavlic *et al.* 2008; Abdollahzadeh *et al.* 2010; Begoude *et al.* 2010; Ismail *et al.* 2012; Liu *et al.* 2012; Urbez-Torres *et al.* 2012; Machado *et al.* 2014; Netto *et al.* 2014; Prasher and Singh 2014; Chen *et al.* 2015; Linaldeddu *et al.* 2015; Trakunyingcharoen *et al.* 2015). It has also become clear that some of the reports of *L. theobromae* prior to 2004 represent other species of *Lasiodiplodia* and a new list of host species for this fungus is required.

Lasiodiplodia plurivora was the first cryptic species to be described in *Lasiodiplodia* (Damm *et al.* 2007), based on sequence variation in the internal transcribed spacer of the rDNA (ITS) and translation elongation factor-1α (*tef*1-α) regions. Shortly thereafter Alves *et al.* (2008) described *Lasiodiplodia parva* and *Lasiodiplodia pseudotheobromae* using the same loci. Subsequently, 20 additional species have been described in the *L. theobromae* complex. The majority of the 24 species that are now known in this complex cannot be identified based on morphology alone. Five species consistently group outside the *L. theobromae* species complex, namely *Lasiodiplodia crassispora*, *Lasiodiplodia gonubiensis*, *Lasiodiplodia pyriformis*, *Lasiodiplodia rubropurpurea*, and *Lasiodiplodia venezuelensis* (Pavlic *et al.* 2004; Burgess *et al.* 2006; Slippers *et al.* 2014).

The PSR concept provides the most powerful means to distinguish between taxa, also in terms of practical uses in quarantine and disease management. Unfortunately this approach is not without problems, especially where only a few loci are used. For example, hybridisation cannot always be recognised if sequences of only one (and often even two) loci have been considered. This is an important consideration because many fungi have the capacity to hybridize through sexual reproduction or exchange genetic material through anastomosis (fusion) of their vegetative hyphae in a parasexual cycle (Olson & Stenlid 2002; Schardl & Craven 2003; Stukenbrock 2016).

There are different possible outcomes of hybridisation in fungi, but only the two outcomes most applicable to this study will be discussed. The first and probably most common is introgression, where the hybrids in the population transfer novel genes to the parent population through backcrosses and the hybrid isolates eventually disappear from the population (Brasier 1995). The second outcome is the establishment of hybrid species that remain stable in the environment (Brasier 1995). These species are then described as *nothospecies* and indicated as hybrids with the symbol '×' as was done for *M. ×columbiana* (Newcombe *et al.* 2000), *Phytophthora ×alni*, *Phytophthora ×multiformis* (Husson *et al.* 2015), and *Phytophthora ×pelgrandis* (Nirenberg *et al.* 2009). It is important to indicate when a new species being described is a hybrid as these species can cause incongruence between different trees of different loci (Schardl & Craven 2003).

Lasiodiplodia occurs globally on woody plants in the tropics and sub-tropics (Punithalingam 1976). Species in the genus have been associated with many different plant diseases including fruit and root rots, die-back of branches and stem cankers (Burgess *et al.* 2006; Sakalidis *et al.* 2011a; Ismail *et al.* 2012; Urbez-Torres *et al.* 2012). *Lasiodiplodia* species have many different plant hosts, but pertinent to this study, they are also well-known on the iconic Baobab (*Adansonia* species), native to Africa and Australia (Roux 2002; Sakalidis *et al.* 2011a). In pathogenicity tests on the Australian baobab (*Adansonia gregorii*), *Lasiodiplodia iraniensis* and *Lasiodiplodia mahajangana* were shown to cause stem lesions and root rot (Sakalidis *et al.* 2011a).

The aims of this study were to identify species of *Lasiodiplodia* on baobab trees in Africa and to assess their ability to cause disease. We also evaluated the suitability of using sequence data from different nuclear loci for species delimitation in *Lasiodiplodia*. Using this information, all species in the genus were reassessed. The possible occurrence of hybrid *Lasiodiplodia* isolates from baobab trees, as well as in the previously described species was a specific focus.

Materials and methods

Sample collection and isolations

South Africa

Plant tissue samples from which to isolate endophytic *Botryosphaeriaceae* from baobab trees (*Adansonia digitata* s.l.) were collected during three surveys conducted in the Limpopo

Province of South Africa (Fig 1, Table 1). The first collections were made in the Soutpansberg and Musina areas in June 2007 and this was followed by sampling in the Venda area and Kruger National Park (KNP) in February 2009. A third collection was made in April 2010 and this extended from the Musina area towards the west and south. Endophyte isolations from the first collection trip were made after surface disinfestation of branch tissue with 5 % HOCl, rinsing in sterile distilled H_2O, disinfesting with 70 % EtOH and again rinsing in sterile distilled H_2O, each for 1 min. Branch samples were then cut into approximately 5 × 5 mm pieces and plated onto 2 % MEA amended with streptomycin. Surface disinfestation of samples collected during the second and third surveys was done by immersing plant tissue in 5 % H_2O_2 for 5 min, followed by rinsing three times in sterile H_2O for 1 min each, after which the samples were cut and plated as described above.

Botswana and Namibia

Branch samples were collected from 12 and 51 *Adansonia digitata* s.l. trees in Botswana and Namibia, respectively (Fig 1, Table 1), from September–October 2007. Isolations for endophytic fungi were made after surface disinfestation with HOCl and EtOH as described above.

Madagascar

During October 2007, branch and bark samples were collected from five of the seven species of baobab trees occurring in Madagascar. Samples were collected from 77 trees (Fig 1, Table 1) and endophyte isolations were made after surface disinfestation with 5 % H_2O_2.

Cameroon

In December 2009, branch and bark samples were collected from 34 baobab trees in three areas in Cameroon (Fig 1, Table 1) and endophytic fungi were isolated as described above after surface disinfestation with 5 % H_2O_2.

Benin and Senegal

Bark and branch samples were obtained from Dr. Aida Cuni Sanchez in January and August 2008 from Senegal and Benin (Fig 1, Table 1). Endophytic fungi were isolated from these samples after surface disinfestation with 5 % H_2O_2, as described above.

Zimbabwe

Bark samples were collected from ten baobab trees from the northern part of the country during July 2010. Endophytic fungi were isolated from these samples after surface disinfestation with 5 % H_2O_2, as described above.

Mozambique

During August 2010, bark samples were collected from six baobab trees. Endophytic fungi were isolated from these samples after surface disinfestation with 5 % H_2O_2, as described above.

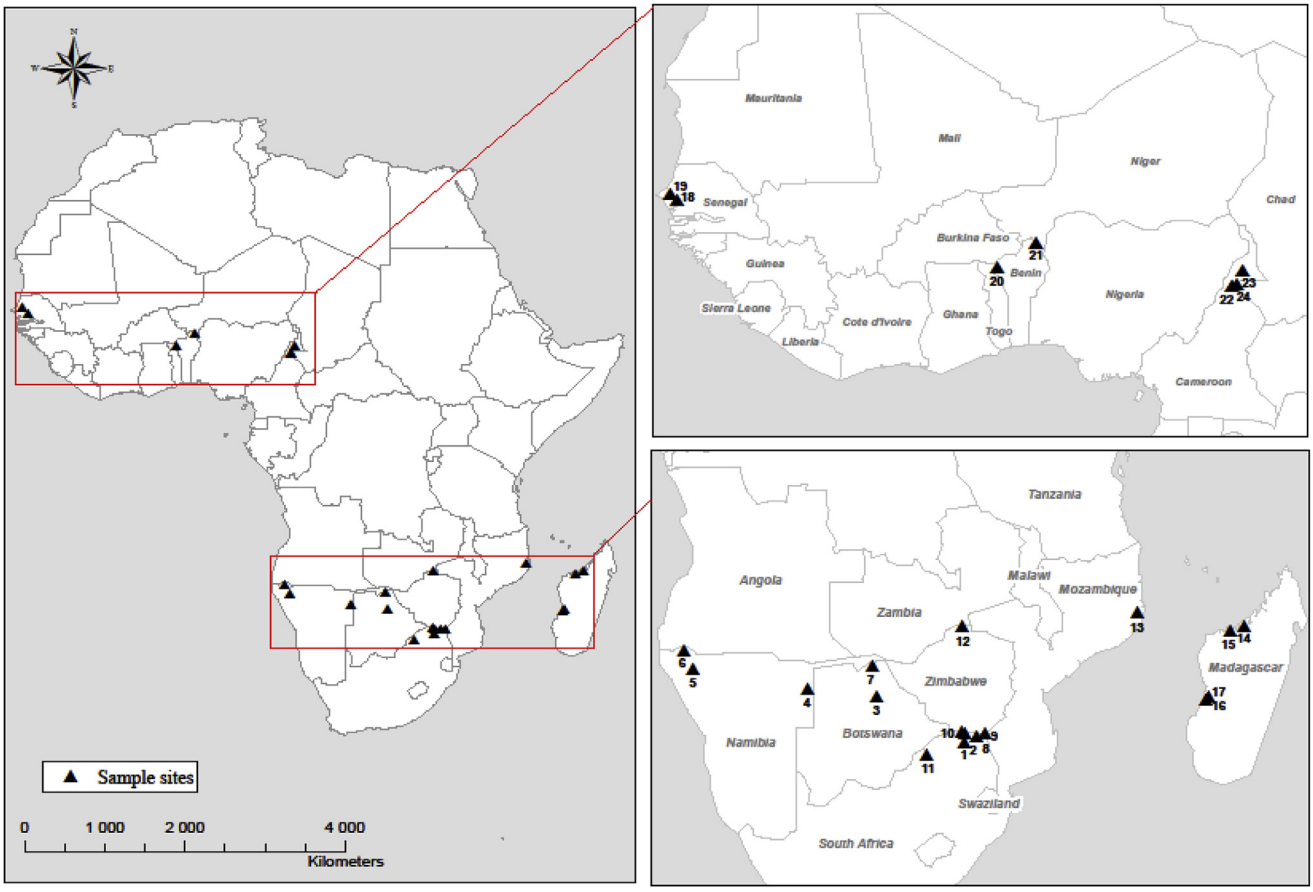

Fig 1 – Map of Africa, indicating areas sampled in southern Africa, West Africa, and Madagascar. Numbers of sample areas correspond to column 3 in Table 1.

Table 1 – Samples collected from baobab trees in southern Africa, West Africa, and Madagascar.

Date	Country	Area on map (Fig 1)	Nr. of trees sampled	Twigs/Bark	Diseased/Healthy
June 2007	South Africa – Musina	1; 2	37	Twigs	Discolouration in wood
Sept. 2007	Botwsana – Nxai pan	3	9	Twigs	Healthy
Oct. 2007	Namibia – Tsumkwe	4	14	Twigs	Many diseased
Oct. 2007	Namibia – Joubert mountains	5	32	Twigs	Healthy
	Namibia – Epupa	6	5	Twigs	Healthy
	Botswana – Chobe	7	3	Twigs	Stressed
Oct. 2007	Madagascar – Andranoboka[a,b]	14	15	Twigs & bark	Not visibly diseased
	Madagascar – Antseza	15	18	Twigs & bark	Not visibly diseased
	Madagascar – Morondava[a,c]	17	16	Bark	Not visibly diseased
	Madagascar – Andranomena[a,d]	16	20	Bark	Not visibly diseased
	Madagascar – Andranomena[a,e]	16	8	Bark	Not visibly diseased
Jan. 2008	Senegal – Fatick	18	1	Twigs & bark	Healthy
			6	Twigs & bark	Diseased
	Senegal – Thies	19	3	Twigs & bark	Healthy
			15	Twigs & bark	Diseased
Feb. 2008	South Africa – Venda	8	14	Twigs	Mostly healthy
	South Africa – Kruger National Park	9	31	Bark	Elephant damage
Aug. 2008	Benin – Materi	20	3	Twigs & Bark	Diseased
	Benin – Bogo bogo	21	1	Bark	Healthy
	Benin		10	Twigs & Bark	Diseased
Dec. 2009	Cameroon – Solawel/Figuil	22	4	Bark	Healthy
	Cameroon – Maroua	23	9	Bark	Healthy
	Cameroon – Lombel	24	21	Bark	Healthy
Apr. 2010	South Africa – Musina area	2	41	Bark	Healthy
	South Africa – Musina-Alldays	10	9	Bark	Healthy
	South Africa – Lephalale	11	5	Bark	Healthy
July 2010	Zimbabwe – Hurungu & Chewore	12	10	Bark	Healthy
Aug. 2010	Mozambique – Monapo	13	6	Bark	Healthy

a *Adansonia* species sampled not *A. digitata*.
b *A. madagascariensis*.
c *A. grandidieri*.
d *A. rubrostipa*.
e *A. za*.

Plates (MEA) were incubated at 25 °C for seven days and checked daily for fungal growth. Pure cultures were made by transferring hyphal tips of the fungi appearing to represent the *Botryosphaeriaceae* to clean MEA plates. Selected isolates from each region were deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

DNA extraction, PCR amplification, and sequencing

Available isolates of previously described *Lasiodiplodia* species (Table 2) were obtained for this study. Isolates of five species described from Brazil (*Lasiodiplodia brasiliense*, *Lasiodiplodia euphorbiicola*, *Lasiodiplodia jatrophicola*, *Lasiodiplodia macrospora*, and *Lasiodiplodia subglobosa*) and one species from India (*Lasiodiplodia indica*) could not be obtained. Isolates of two species that were described during 2015, were also not included. These were *Lasiodiplodia americana* from the United States of America, which has subsequently been reduced to synonymy with *Lasiodiplodia exigua* (Rodríguez-Gálvez *et al.* 2017) and *Lasiodiplodia thailandica* (Trakunyingcharoen *et al.* 2015) from Thailand. The ex-type isolate of *Lasiodiplodia laeliocattleyae* was not included in this study, however the ex-type isolate of *Lasiodiplodia egyptiacae*, which was recently reduced to synonymy with *L. laeliocattleyae* (Rodríguez-Gálvez *et al.* 2017), was included.

All isolates, including those from baobabs, were grown for 7 d at 25 °C on 2 % MEA, after which mycelium was scraped from the surfaces of the medium and freeze dried. Freeze dried mycelium was ground to a powder and DNA was extracted as described by Möller *et al.* (1992). DNA was amplified with PCR using commonly applied primers (Table 3).

The ITS and *tef*1-α gene regions were amplified for all *Lasiodiplodia* isolates from baobab trees. A sub-set of isolates from different geographic areas with different ITS and *tef*1-α sequences were further characterised by amplifying and sequencing the β-tubulin 2 (*tub*2) and RNA polymerase subunit II (*rpb*2) gene regions. The *tub*2, calmodulin (*cmdA*) and *rpb*2 gene regions were also sequenced for all available isolates of previously described species. New primers (Table 3) were developed for the *rpb*2 region, because the primers normally used for the *Botryosphaeriaceae* were not effective for *Lasiodiplodia*. The new forward primer binds at the same position as the primer developed by Sakalidis *et al.* (2011b) with one base pair that was changed. The reverse primer binds four base pairs away from the primer developed by Sakalidis *et al.* (2011b).

All amplification reactions consisted of 1.5 U MyTaq™ DNA Polymerase (Bioline, London, UK), 5 μL MyTaq PCR reaction buffer, 0.2 μM of each primer and 50 ng template DNA (made up to a total volume of 25 μL with PCR grade water). PCR conditions were 2 min at 95 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 52–54 °C (depending on gene region), 1 min at

Table 2 – Isolates of existing *Lasiodiplodia* species included in analyses.

Species	Isolate no.	CMW no.	Mycobank	Country	Host	GenBank accession numbers				
						ITS	*tef1-α*	*tub2*	*cmdA*	*rpb2*
L. brasiliense	CMM 4015[a]		MB807525	Brazil	*Mangifera indica*	JX464063	JX464049			
	CMM 2320			Brazil	*Carica papaya*	KC484814	KC481544			
		CMW 35884		Madagascar	*Adansonia madagascariensis*	KU887094	KU886972	KU887466	KU886755	KU696345
L. citricola	CBS 124707[a]	CMW 37046	MB16777	Iran	*Citrus* sp.	GU945354	GU945340	KU887505	KU886760	KU696351
	CBS 124706	CMW 37047		Iran	*Citrus* sp.	GU945353	GU945339	KU887504	KU886759	KU696350
L. crassispora	CBS 118741[a]	CMW 14691	MB500235	Australia	*Santalum album*	DQ103550	DQ103557	KU887506	KU886761	KU696353
		CMW 13488		Venezuela	*Eucalyptus urophylla*	DQ103552	DQ103559	KU887507	KU886762	KU696352
L. euphorbiicola	CMM 3609[a]		MB804872	Brazil	*Jatropha curcas*	KF234543	KF226689	KF254926		
	CMM 3651			Brazil	*J. curcas*	KF234553	KF226711	KF254937		
		CMW 33350		Botswana	*A. digitata*	KU887149	KU887026	KU887455	KU886754	KU696346
		CMW 36231		Zimbabwe	*A. digitata*	KU887187	KU887063	KU887494	KU886756	KU696347
L. exigua	CBS 137785[a]	CMW 43391	MB808355	Tunisia	*Retama raetam*	KJ638317	KJ638336	KU887509	KU886764	KU696355
	PD 161			USA	*Pistachia vera*	GU251122	GU251254			
(*L. americana*)	CERC 1961[a]		MB810934	USA	*P. vera*	KP217059	KP217067	KP217075		
	CERC 1960			USA	*P. vera*	KP217058	KP217066	KP217074		
L. gonubiensis	CBS 115812[a]	CMW 14077	MB500079	South Africa	*Syzigium cordatum*	DQ458892	DQ458877	DQ458860	KU886768	KU696359
	CBS 116355	CMW 14078		South Africa	*S. cordatum*	AY639594	DQ103567	EU673126	KU886767	KU696358
L. hormozganensis	CBS 124709[a]	CMW 37050	MB16779	Iran	*Olea* sp.	GU945355	GU945343	KU887515	KU886770	KU696361
	CBS 124708	CMW 40931		Iran	*M. indica*	GU945356	GU945344	KU887514	KU886769	KU696360
L. indica	IBP 01		MB810909	India	wood	KM376151				
L. iraniensis	CBS 124710[a]	CMW 37051	MB16780	Iran	*Salvadora persica*	GU945348	GU945336	KU887516	KU886771	KU696363
	CBS 124711	CMW 37052		Iran	*Juglans* sp.	GU945347	GU945335	KU887517	KU886772	KU696362
(*L. jatrophicola*)	CMM 3610[a]		MB804869	Brazil	*J. curcas*	KF234544	KF226690	KF254927		
		CMW 36237		Mozambique	*A. digitata*	KU887121	KU886998	KU887499	KU886757	KU696348
		CMW 36239		Mozambique	*A. digitata*	KU887123	KU887000	KU887501	KU886758	KU696349
L. laeliocattleyae	CBS 130992[a]	CMW 40930	MB564516	Egypt	*M. indica*	JN814397	JN814424	KU887508	KU886763	KU696354
(*L. egyptiacae*)	BOT-29			Egypt	*M. indica*	JN814401	JN814428			
L. lignicola	CBS 134112[a]	CMW 40932	MB801317	Thailand	dead wood	JX646797	KU887003	JX646845		KU696364
	MFLUCC 11-0656		MB805462	Thailand	dead wood	JX646798		JX646846		
L. macrospora	CMM 3833[a]		MB804871	Brazil	*J. curcas*	KF234557	KF226718	KF254941		
L. mahajangana	CBS 124925[a]	CMW 27801	MB514012	Madagascar	*Terminalia catappa*	FJ900595	FJ900641	KU887518	KU886773	KU696365
	CBS 124926	CMW 27818		Madagascar	*T. catappa*	FJ900596	FJ900642	KU887519	KU886774	KU696366
L. margaritacea	CBS 122519[a]	CMW 26162	MB512052	Australia	*A. gregorii*	EU144050	EU144065	KU887520	KU886775	KU696367
L. mediterranea	CBS 137783[a]	CMW 43392	MB808356	Italy	*Quercus ilex*	KJ638312	KJ638331	KU887521	KU886776	KU696368
	CBS 137784	CMW 43393		Italy	*Vitis vinifera*	KJ638311	KJ638330	KU887522	KU886777	KU696369
L. missouriana	CBS 128311[a]	CMW 40933	MB519954	USA	*Catawba*	HQ288225	HQ288267	HQ288304	KU886778	KU696370
	CBS 128312	CMW 40934		USA	*Catawba*	HQ288226	HQ288268	HQ288305	KU886779	KU696371
L. parva	CBS 456.78[a]	CMW 40935	MB510942	Colombia	cassava field soil	EF622083	EF622063	KU887523	KU886780	KU696372
	CBS 494.78	CMW 40936		Colombia	cassava field soil	EF622084	EF622064	EU673114	KU886781	KU696373
L. plurivora	CBS 120832[a]	CMW 40937	MB501322	South Africa	*Prunus salicina*	EF445362	EF445395	KU887524	KU886782	KU696374
	CBS 121103	CMW 40938		South Africa	*V. vinifera*	AY343482	EF445396	KU887525	KU886783	KU696375
L. pseudotheobromae	CBS 116459[a]	CMW 40939	MB510941	Costa Rica	*Gmelina arborea*	EF622077	EF622057	EU673111	KU886784	KU696376
		CMW 9074		Mexico	*Pinus* sp.	AY236952	AY236901	KU887526	KU886785	KU696377
L. pyriformis	CBS 121770[a]	CMW 25414	MB518722	Namibia	*Acacia mellifera*	EU101307	EU101352	KU887527	KU886786	KU696378
	CBS 121771	CMW 25415		Namibia	*A. mellifera*	EU101308	EU101353	KU887528	KU886787	KU696379

L. rubropurpurea	CBS 118740[a]	CMW 14700	MB500236	Australia	*E. grandis*	DQ103553	DQ103571	EU673136	KU886788	KU696380
	WAC 12536	CMW 15207		Australia	*E. grandis*	DQ103554	DQ103572	KU887530		KU696381
L. subglobosa	CMM 3872[a]		MB804870	Brazil	*J. curcas*	KF234558	KF226721	KF254942		
	CMM 4046			Brazil	*J. curcas*	KF234560	KF226723	KF254944		
L. thailandica	CBS 138760		MB810169	Thailand	*M. indica*	KP217058	KP217066			
	CBS 138653			Thailand	*Phyllanthus acidus*	KJ193637	KJ193681			
L. theobromae	CBS 164.96[a]	CMW 40942	MB188476	New Guinea	Fruit on coral reef coast	AY640255	AY640258	KU887532	KU886789	KU696383
	CBS 111530	CMW 40953		Unknown	Unknown	EF622074	EF622054	KU887531	KU886790	KU696382
L. venezuelensis	CBS 118739[a]	CMW 13511	MB500237	Venezuela	*A. mangium*	DQ103547	DQ103568	KU887533	KU886791	KU696384
	WAC 12540	CMW 13512		Venezuela	*A. mangium*	DQ103548	DQ103569	KU887534	KU886792	
L. viticola	CBS 128313[a]	CMW 40944	MB519955	USA	hybrid grape *Vignoles*	HQ288227	HQ288269	HQ288306	KU886793	KU696385
	CBS 128314	CMW 41372		USA	*Chardonel*	HQ288228	HQ288270	HQ288307	KU886794	KU696386
Botryosphearia dothidea	CBS 115476	CMW 8000		Switzerland	*Prunus* sp.	KF766151	AY236898			DQ677944

BOT: A. M. Ismail, Plant Pathology Research Institute, Egypt.
CERC: Culture collection of China Eucalypt Research Centre, Chinese Academy of Forestry, ZhanJiang, GuangDong, China.
CMM: Culture Collection of Phytopathogenic Fungi 'Prof. Maria Menezes', Universidade Federal Rural de Pernambuco, Recife, Brazil.
CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.
CBS: Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
MFLUCC: Mae Fah Luang University Culture Collection, ChiangRai, Thailand.
WAC: Department of Agriculture Western Australia Plant Pathogen Collection, South Perth, Western Australia.
a Ex-type strain.

72 °C, and a last extension step of 8 min at 72 °C. PCR products were visualised on a 1 % agarose gel stained with GelRed (Biotium, Hayward, California, USA) and successful PCR products were purified with Exosap (Mixture of Exonuclease I and FastAP Alkaline Phosphatase) (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's specifications.

DNA sequencing was conducted with the ABI Prism® Big Dye™ Terminator 3.1 Ready Reaction Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequences were determined with an ABI PRISM™ 3100 Genetic Analyzer (Applied Biosystems) at the University of Pretoria. The same primer sets as those used for PCR amplification were utilised. Forward and reverse sequences were assembled with CLC Main workbench v.6.1 (CLC Bio, www.clcbio.com).

Phylogenetic analyses

Sequences of the type strains of all *Lasiodiplodia* species on GenBank (http://www.ncbi.nlm.nih.gov) were downloaded and aligned with newly generated sequences using the MAFFT v.7 server (http://mafft.cbrc.jp/alignment/server/) and manually adjusted where necessary. *Botryosphaeria dothidea* was used as the outgroup taxon in all analyses other than for *cmdA*, which was midpoint rooted. This exception was necessary because there were no closely related sequences for *cmdA* available on GenBank. Individual trees of existing species were first generated and the best substitution models were determined for each dataset with jModeltest v.2.1.3 using the Akaike Information Criterion (AIC) (Guindon & Gascuel 2003; Darriba *et al.* 2012). Maximum Likelihood (ML) analyses were done with PhyML v.3.0 (Guindon & Gascuel 2003) and 1000 bootstrap replicates were run to determine confidence levels for the branches. PHYLIP v.3.6 (Felsenstein 2005) was used to generate consensus trees using the *consense* option. Maximum parsimony (MP) analyses were performed using PAUP v.4.0 beta 10 (Swofford 2003) with Tree Bisection-Reconnection (TBR), with ten trees saved per replicate and with 1000 bootstrap replicates. Bayesian inference, based on a Markov Chain Monte Carlo (MCMC) approach, was performed in MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003), with 1 000 000 generations, sampled every 100 generations. Burnin values were determined using Microsoft Excel 2013. All sampled trees having lower values than the burn-in were discarded.

Re-evaluation of existing *Lasiodiplodia* species identified hybrid isolates and species. These were not included in further analyses. A combined dataset of tub2, ITS, *tef*1-α and *rpb*2 was generated to identify the species from baobabs. The same analyses were applied as described above to generate phylogenetic trees.

Pathogenicity trials

Baobab seeds (*Adansonia digitata* s.l.) were treated with hot water overnight and placed in germination trays with a mixture of sand, top soil and potting soil. After germination, the seedlings were transplanted into larger containers in a mixture of sand: top soil: potting soil (50:25:25). Trees were grown in containers for three years.

Lasiodiplodia isolates of different species and from different regions were selected to test whether any of the species are

Table 3 – Primers used to amplify selected gene regions.

Primer name	Sequence	Reference
ITS1-F	5′-CTTGGTCATTTAGAGGAAGTAA-3′	Gardes & Bruns (1993)
ITS4	5′-TCCTCCGCTTATTGATATGC-3′	White *et al.*(1990)
EF1-688F	5′-CGGTCACTTGATCTACAAGTGC-3′	Alves *et al.* (2008)
EF1-1251R	5′-CCTCGAACTCACCAGTACCG-3′	Alves *et al.* (2008)
Bt2a	5′-GGTAACCAAATCGGTGCTGCTTTC-3′	Glass & Donaldson (1995)
Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	Glass & Donaldson (1995)
rpb2-LasF	5′-GGTAGCGACGTCACTCCT-3′	This study
rpb2-LasR	5′-GCGCAAATACCCAGAATCAT-3′	This study
CAL-228F	5′-GAGTTCAAGGAGGCCTTCTCCC-3′	Carbone & Kohn (1999)
CAL-737R	5′-CATCTTTCTGGCCATCATGG-3′	Carbone & Kohn (1999)

pathogenic to baobab trees. Variability in virulence between isolates of the species that were most commonly isolated from baobab trees was also tested. A total of 13 *Lasiodiplodia* isolates including *Lasiodiplodia euphorbiicola* (3 isolates), *Lasiodiplodia iraniensis* (1 isolate), *Lasiodiplodia jatrophicola* (1 isolate), *Lasiodiplodia mahajangana* (6 isolates), and *Lasiodiplodia pseudotheobromae* (2 isolates) were selected from amongst isolates from baobabs in southern Africa for use in pathogenicity trials. Isolates were grown on 2 % MEA for 7 d. A 5 mm-diameter cork borer was used to cut holes approximately 5 mm deep in the stems of the trees about 10 cm above ground level. Using the same size cork borer, discs of agar covered in mycelium were cut from actively growing cultures (one-week-old) and these were placed in the wounds on the plant stems. The inoculation sites were sealed with parafilm to minimize desiccation and to reduce chances of contamination. A randomised block design was generated with www.randomization.com and ten replicates per treatment were used. Non-colonised 2 % MEA was used for the controls. The trial was left for six weeks after which the lesions were measured and fungi re-isolated. Statistical significance of the data was determined with a single factor ANOVA followed by a Duncan multiple range test in Microsoft Excel 2010.

Results

Sample collection and isolation

Endophyte isolations yielded a total of 420 isolates that resembled *Lasiodiplodia* species based on culture morphology. A total of 130 isolates were obtained from South Africa, 26 from Botswana, 30 from Namibia, 5 from Zimbabwe, 7 from Mozambique and 104 from Madagascar. From West Africa 59 isolates were obtained from Cameroon, 30 from Senegal and 29 from Benin. Of these, 320 were selected for further identification by DNA sequencing, after excluding multiple isolates from the same trees. The isolations from tissue samples that had been surface disinfested with H_2O_2 yielded more than double the number of *Lasiodiplodia* cultures than those where HOCl and EtOH were used. This may account for the low numbers of isolates obtained from Namibia and Botswana and from the first sampling trip in South Africa (20 isolates).

Phylogenetic analyses

Alignment of sequences for previously described species yielded datasets of 461 bp, 517 bp, 423 bp, 532 bp, and 510 bp for the ITS, *tef*1-α, tub2, *rpb*2, and *cmd*A, respectively. The alignment of the *tef*1-α sequences was the most problematic, due to a large amount of variability within the intron 3 region, and minor manual adjustments were made where necessary. The *tef*1-α sequence for *Lasiodiplodia lignicola* on GenBank (JX646862) did not group within *Lasiodiplodia* and is deemed to be an incorrect sequence for this isolate. A new *tef*1-α sequence (KU887003) was generated from the ex-type strain and used in analyses.

Phylogenetic analyses of sequences from the ITS (Fig 2A) and *tub*2 (Fig 2B) loci did not differentiate between all *Lasiodiplodia* species. Analyses of *rpb*2 sequences (Fig 2C) could distinguish between most *Lasiodiplodia* species other than *Lasiodiplodia parva* and *Lasiodiplodia euphorbiicola*, and *Lasiodiplodia brasiliense*, *Lasiodiplodia laeliocattleyae*, *Lasiodiplodia theobromae*, as well as *Lasiodiplodia mediterranea*, *Lasiodiplodia missouriana*, and *Lasiodiplodia viticola*. The most variable locus was *tef*1-α (Fig 2D) which could distinguish between most species, but not between *L. brasiliense* and *L. viticola* or between *Lasiodiplodia iraniensis* and *Lasiodiplodia jatrophicola*. The *cmd*A (Fig 2E) dataset appeared to distinguish between species better than ITS and *tub*2. None of the loci tested could distinguish between all of the currently described species and a combination of loci was, therefore, needed to identify *Lasiodiplodia* to species level.

The trees from individual loci (Fig 2) for previously described species showed concordance between *tub*2, *cmd*A, ITS, and *rpb*2. In the *tef*1-α tree, some species failed to show the same groupings found in the other phylogenetic trees and were not considered congruent. These included the *L. theobromae* group (including *L. brasiliense* and *L. laeliocattleyae*), *Lasiodiplodia pseudotheobromae* group (including *L. iraniensis* and *L. jatrophicola*) and the *L. mediterranea* group (including *L. missouriana* and *L. viticola*). The incongruence of the species in the *tef*1-α tree could be explained only by accepting that some of these species, as represented by the ex-type isolates, were hybrids.

Based on the ITS dataset, *L. theobromae* was identical to *L. laeliocattleyae*, *L. brasiliense*, and *Lasiodiplodia hormozganensis*; *tub*2, *cmd*A, and *rpb*2 also grouped *L. theobromae* and *L.*

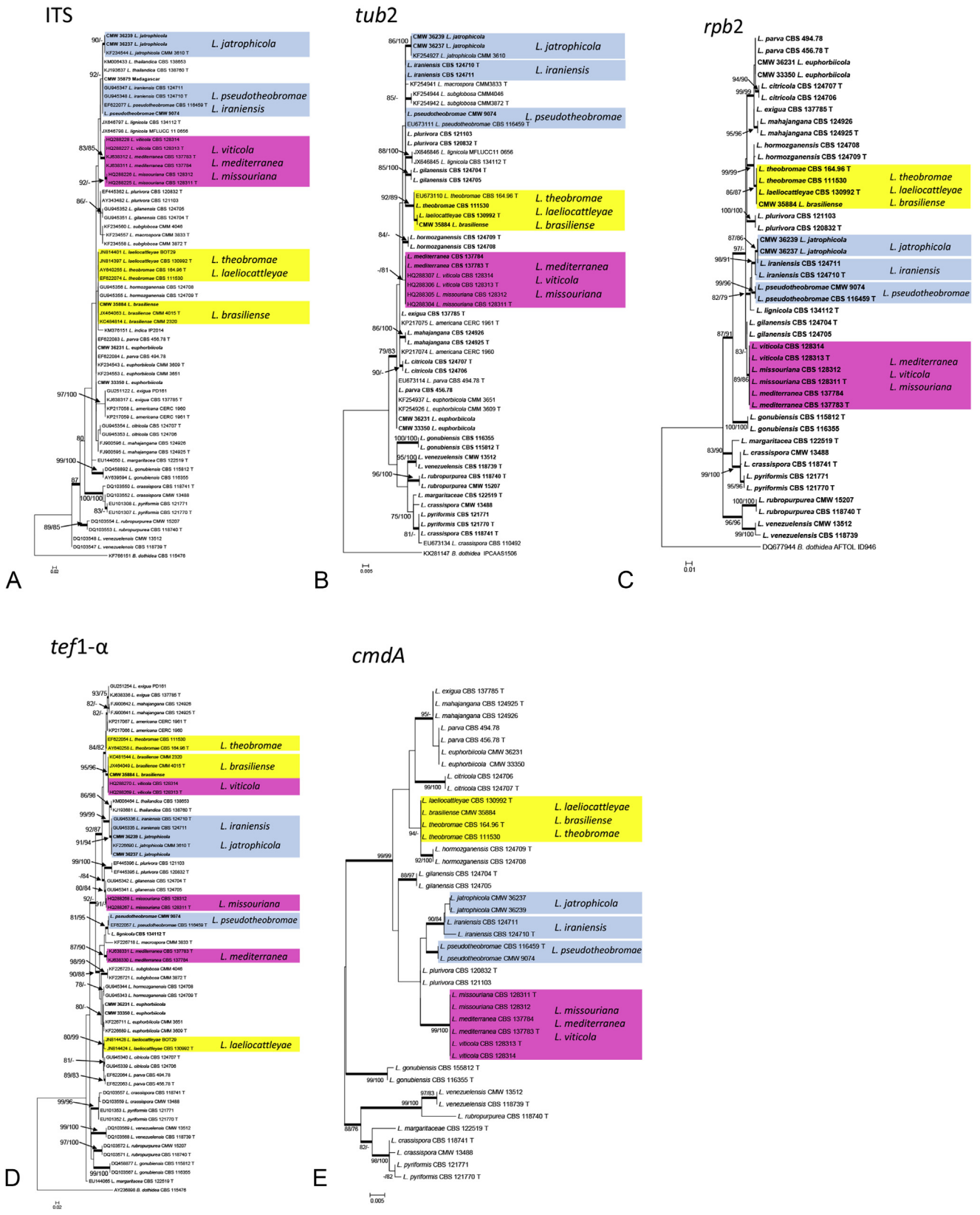

Fig 2 – Maximum likelihood trees of currently described *Lasiodiplodia* species based on partial (A) ITS, (B) *tub*2, (C) *rpb*2, (D) *tef*1-*α*, and (E) *cmdA* gene sequences. Sequences in bold, as well as all *cmdA* sequences, were obtained during this study. Bootstrap values above 70 % (indicated as ML/MP) are given at the nodes. Branches with Bayesian posterior probabilities of more than 0.95 are printed in bold. Trees A–D were rooted with *B. dothidea* and E was midpoint rooted.

laeliocattleyae together. An ex-type isolate of *L. brasiliense* was not available to generate *tub*2, *cmd*A, and *rpb*2 sequences, but an isolate (CMW 35884) from baobab grouped with the ex-type isolate of this species based on ITS and *tef*1-α, and this isolate showed similarity to *L. theobromae* on *tub*2, *cmd*A, and *rpb*2. *Lasiodiplodia hormozganensis* was a sister species to *L. theobromae* based on *tub*2, *cmd*A, and *rpb*2, but not based on *tef*1-α.

Lasiodiplodia pseudotheobromae grouped with *L. iraniensis* based on ITS and *tub*2, while *rpb*2 and *cmd*A separated *L. pseudotheobromae* from *L. iraniensis*, but still grouped them as sister species. While *tef*1-α also separated *L. pseudotheobromae* from *L. iraniensis*, it did not group them as sister species. The *tef*1-α locus also did not distinguish *L. jatrophicola* from *L. iraniensis*, as occurs with the ITS, *tub*2, *cmd*A, and *rpb*2 sequences. Although an ex-type isolate of *L. jatrophicola* was not available for *rpb*2 and *cmd*A sequencing, several isolates from baobab trees (CMW 36237, CMW 36239) grouped with the ex-type isolate of this species based on its *tef*1-α and ITS sequences.

Lasiodiplodia iraniensis showed some variability within the species based on *rpb*2 and *cmd*A sequences. The ex-type isolate of *L. iraniensis* (CBS 124710) consistently grouped separate from *L. jatrophicola* based on ITS, *tub*2, *cmd*A, and *rpb*2. However, the paratype isolate (CBS 124711) grouped with the ex-type isolate in ITS, but grouped between *L. iraniensis* and *L. jatrophicola* based on *cmd*A, and was identical to *L. jatrophicola* based on *rpb*2. This may indicate gene flow and supports the synonymy of *L. jatrophicola* with *L. iraniensis* based on a phylogeny of combined ITS and *tef*1-α data by Rodríguez-Gálvez *et al.* (2017).

When considering the *tub*2, *rpb*2, and *cmd*A sequences *L. missouriana*, *L. mediterranea*, and *L. viticola* were identical. Although ITS separated the three species, it grouped them in a single clade. The *tef*1-α locus grouped the three species close to three other unrelated species and not as sister species, as would be expected based on the *tub*2, *rpb*2, *cmd*A, and ITS loci.

The trees from the individual loci in conjunction with a decision tree (Fig 3) were used to determine which of the currently described species are hybrids, and this approach was also taken for the isolates from baobab trees. To give one example, isolate CMW 33342 grouped with *Lasiodiplodia mahajangana* based on ITS and *tef*1-α and with *L. euphorbiicola* based on *tub*2 and *rpb*2. This would place it in the category of multiple incongruent genes that grouped it with non-sister species and it is, therefore, identified as a hybrid.

The ex-type strains of *L. brasiliense*, *L. laeliocattleyae*, *L. missouriana*, and *L. viticola* displayed incongruence between all other loci and *tef*1-α, grouping with distant species in the phylogenies of different loci. Following the logic provided by the decision tree in Fig 3, these isolates were considered hybrids. The species names are consequently invalid and they are designated here as hybrid species. All isolates identified as *L. brasiliense*, *L. laeliocattleyae*, *L. missouriana*, and *L. viticola* were identified based on *tef*1-α, which is where the incongruence with other genes emerge and as such they must also be hybrids. Isolates from baobab trees that appeared to be hybrids are not described as hybrid species because they could be transient hybrid isolates that may yet disappear.

Taxonomy

Based on comparison of ITS, *tef*1-α, *tub*2, *rpb*2, and *cmd*A gene regions for the ex-type isolates, *L. laeliocattleyae*, *L. brasiliense*, *L. missouriana*, and *L. viticola* are designated as hybrid species and are described as follows:

Lasiodiplodia ×laeliocattleyae A.M. Ismail, L. Lombard & Crous **nothosp.**, *Australas. Plant Path.* **41**: 655 (2012).

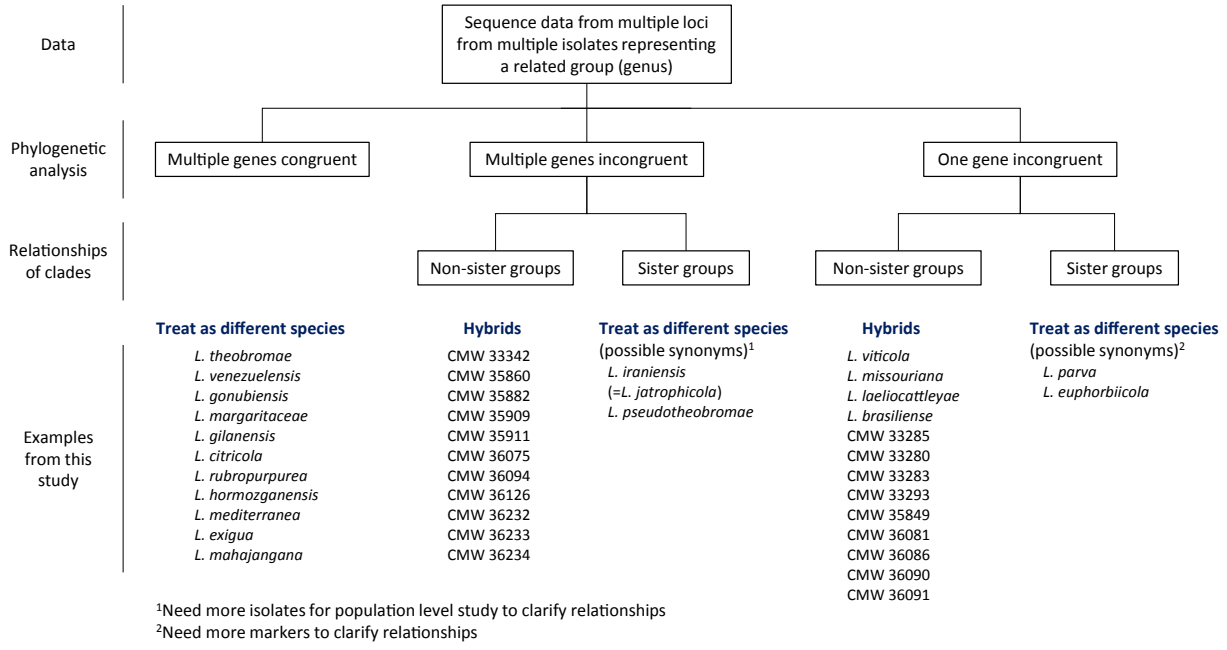

Fig 3 – Decision tree used together with multiple single gene phylogenies to identify hybrids amongst *Lasiodiplodia* isolates. Species and isolates used as examples correlate to Fig 2 and Table 4.

MycoBank MB564516

Lasiodiplodia laeliocattleyae was described from the *Laeliocattleya* orchid in Italy (Rodríguez-Gálvez *et al.* 2017) and has also been reported from *Mangifera indica* (Mango) in Egypt, *Jatropha curcas* in Brazil (Machado *et al.* 2014) and *Adansonia grandidieri* in Madagascar (this study). This species has conidial sizes that overlap with those of *L. theobromae*, although the conidia of *L. laeliocattleyae* are slightly smaller than those reported for *L. theobromae*. DNA sequences of *L. theobromae* and *L. laeliocattleyae* are identical based on ITS, *rpb2*, and *cmdA* and there is a one base pair difference in the *tub2* gene between *L. theobromae* and *L. laeliocattleyae* sequences. However, the *tef1-α* sequences of these two species group them as distantly related, non-sister groups. Therefore, *L. laeliocattleyae* is considered a hybrid of *L. theobromae* and another species, possibly *L. parva* or *L. citricola*.

Lasiodiplodia ×brasiliense M.S.B. Netto, M.W. Marques & A.J.L. Phillips **nothosp.**, Fungal Divers. **67**: 134 (2014).
MycoBank MB807525

Lasiodiplodia brasiliense was described from *Carica papaya* and *M. indica* in Brazil (Netto *et al.* 2014). It has also been reported from *Tectona grandis* in Thailand (Doilom *et al.* 2015), strawberries in Turkey (Yildiz *et al.* 2014) and *A. madagascariensis* in Madagascar (this study). The conidial sizes of *L. theobromae* and *L. brasiliense* overlap, although the conidia of *L. brasiliense* are slightly smaller than those reported for *L. theobromae*. Based on ITS, *rpb2*, and *cmdA* sequences *L. theobromae* and *L. brasiliense* are identical, while there is only one base pair difference between them in sequences of the *tub2* locus. Based on the *tef1-α* dataset, *L. brasiliense* is identical to *L. viticola* and groups as a sister species to *L. theobromae*. The hybrid *Lasiodiplodia ×brasiliense* described here could have arisen from hybridisation between *L. theobromae* and another currently unknown species.

Lasiodiplodia ×missouriana J.R. Úrbez-Torres, F. Peduto & W.D. Gubler **nothosp.** *Fungal Divers.* **52**: 181 (2012).
MycoBank MB519954

Lasiodiplodia missouriana was described from grape cultivars in the USA (Urbez-Torres *et al.* 2012). In the current study *L. missouriana* grouped with *L. mediterranea* and the hybrid species *L. viticola* based on *tub2*, *cmdA*, ITS, and *rpb2* sequences, but based on *tef1-α* it grouped with *L. gilanensis* with only one base pair difference. Therefore, isolates of the hybrid species *L. ×missouriana* described here appear to have arisen through a hybridisation between *L. mediterranea* and *L. gilanensis*.

Lasiodiplodia ×viticola J.R. Úrbez-Torres, F. Peduto & W.D. Gubler **nothosp.** *Fungal Divers.* **52**: 183. 2012.
MycoBank MB519955

Lasiodiplodia viticola was described from grape cultivars (Urbez-Torres *et al.* 2012), and has also been found on *M. indica* in Brazil (Marques *et al.* 2013). Based on *tub2*, *cmdA*, ITS and *rpb2* sequences for the ex-type isolate the hybrid species *L. ×viticola*, defined here, groups with *L. mediterranea* and hybrid species *L. ×missouriana*. However, based on *tef1-α* it is identical to hybrid species *L. ×brasiliense* that is closely related to *L. theobromae*, as discussed above. Isolates of *Lasiodiplodia ×viticola* have probably arisen from hybridization between *L. mediterranea* and *L. theobromae*. Grape is a known host of *L. theobromae* (Úrbez-Torres & Gubler 2009) and also of *L. mediterranea* (Linaldeddu *et al.* 2015) and co-infection of this host by the two species may have provided the opportunity for the hybridization.

Identification of isolates from baobab trees

The individual trees for ITS, *tef1-α*, *tub2*, and *rpb2* sequence datasets for the baobab isolates were compared and 30 hybrid isolates from baobabs excluded (Table 4). The individual trees, as well as a combined dataset for the *tub2*, ITS, *tef1-α*, and *rpb2* sequences were then used to identify *Lasiodiplodia* species from baobab trees. The combined dataset contained 1772 base pairs, of which 1410 characters were constant and 217 characters were parsimony-informative, while 145 variable characters were parsimony uninformative. Maximum Parsimony analyses yielded a tree (Fig 4) having a RI = 0.92, CI = 0.76 and HI = 0.244, and a tree length of 545. The best model selected for Maximum Likelihood analyses for the combined dataset was TrN + G.

Table 4 – Hybrid *Lasiodiplodia* isolates from baobab trees, indicating which species isolates grouped with based on different gene regions.

Isolate	Country	ITS	*tef1-α*	*tub2*	*rpb2*
CMW 33258	Senegal	M	Eu	Eu	Eu
CMW 33280	Benin	M	M	Eu	M
CMW 33283	Benin	M	Eu	Eu	Eu
CMW 33293	Benin	M	Eu	Eu	Eu
CMW 33342	SA	M	M	Eu	Eu
CMW 35849	Madagascar	M	M	Eu	Eu
CMW 35860	Madagascar	M	M	Eu	Eu
CMW 35882	Madagascar	M	M	Eu	Eu
CMW 35909	Madagascar	M	M	Eu	Eu
CMW 35911	Madagascar	M	M	Eu	Eu
CMW 36075	Cameroon	PS/I	M	Eu	M
CMW 36081	Cameroon	PS/I	Eu	Eu	Eu
CMW 36086	Cameroon	Eu	I	PS/I	I
CMW 36090	Cameroon	M	M	Eu	M
CMW 36091	Cameroon	M	Eu	Eu	Eu
CMW 36092	Cameroon	M	EU	Eu	Eu
CMW 36094	Cameroon	Eu	M	M	EX
CMW 36096	Cameroon	Eu	M	Eu	Eu
CMW 36099	Cameroon	M	Eu	Eu	Eu
CMW 36105	Cameroon	Eu	M	Eu	Eu
CMW 36106	Cameroon	M	Eu	Eu	Eu
CMW 36119	Cameroon	EX	Eu	Eu	Eu
CMW 36122	Cameroon	M	Eu	Eu	Eu
CMW 36123	Cameroon	M	I	PS/I	I
CMW 36126	Cameroon	Eu	M	M	Eu
CMW 36232	Zimbabwe	Eu	M	M	Eu
CMW 36233	Zimbabwe	Eu	M	M	Eu
CMW 36234	Zimbabwe	Eu	M	M	Eu

M = *Lasiodiplodia mahajangana*, Eu = *L. euphorbiicola*, Ex = *L. exigua*, I = *L. iraniensis*, PS/I = *L. pseudotheobrome/L. iraniensis* clade.

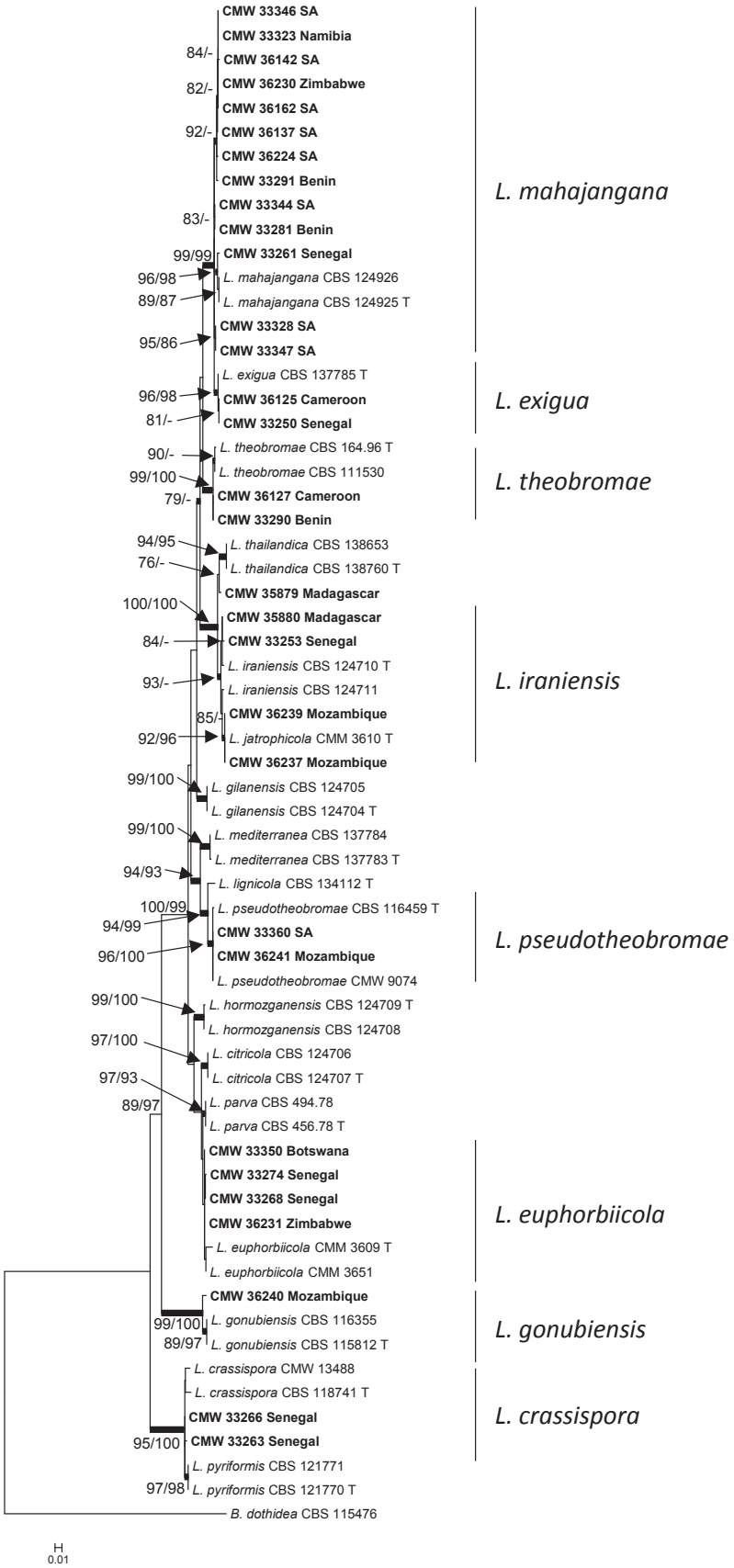

Fig 4 – Maximum likelihood tree of currently described *Lasiodiplodia* species based on partial *tub*2, ITS, *tef*1-α, and *rpb*2 gene regions, tree was rooted with *B. dothidea*. Sequences in bold were obtained during this study. Bootstrap values above 70 % (indicated as ML/MP) are given at the nodes. Branches with Bayesian posterior probabilities of more than 0.95 are printed in bold.

The isolates from baobab trees were identified as *Lasiodiplodia* ×*brasiliense*, *Lasiodiplodia crassispora*, *Lasiodiplodia* ×*laeliocattleyae*, *Lasiodiplodia euphorbiicola*, *Lasiodiplodia exigua*, *Lasiodiplodia gonubiensis*, *Lasiodiplodia iraniensis*, *Lasiodiplodia mahajangana*, *Lasiodiplodia pseudotheobromae*, and *Lasiodiplodia theobromae*. One isolate from Madagascar grouped close to, but distinct from *Lasiodiplodia thailandica* and *L. iraniensis*. The *L. mahajangana* clade included the largest number of isolates (186) and it also had the largest degree of variation within a species, forming four sub-groups. Isolates in these sub-groups did not consistently group together based on different loci and could not be described as new species. This species was found in all countries sampled and appears to be the dominant species in South Africa, Namibia and Madagascar, where it was obtained from both healthy and diseased trees.

The second largest group of isolates (70) grouped with *L. euphorbiicola*. There was less sequence variation between these isolates than within the *L. mahajangana* group, but the same trend was evident where the sub-groups of isolates did not consistently group together based on the different gene regions. *Lasiodiplodia euphorbiicola* was the dominant species isolated from all three West African countries, where it occurred on both healthy and diseased trees. Other countries where it was found included Botswana, Namibia, Madagascar, and Zimbabwe.

Species isolated only from West Africa included *L. exigua* (7 isolates) from Benin, Cameroon and Senegal and the two *L. theobromae* isolates that originated from Benin and Cameroon. *Lasiodiplodia crassispora* was isolated only from Senegal, and was collected from five trees. There was no distinction in the species assemblage from healthy and diseased trees.

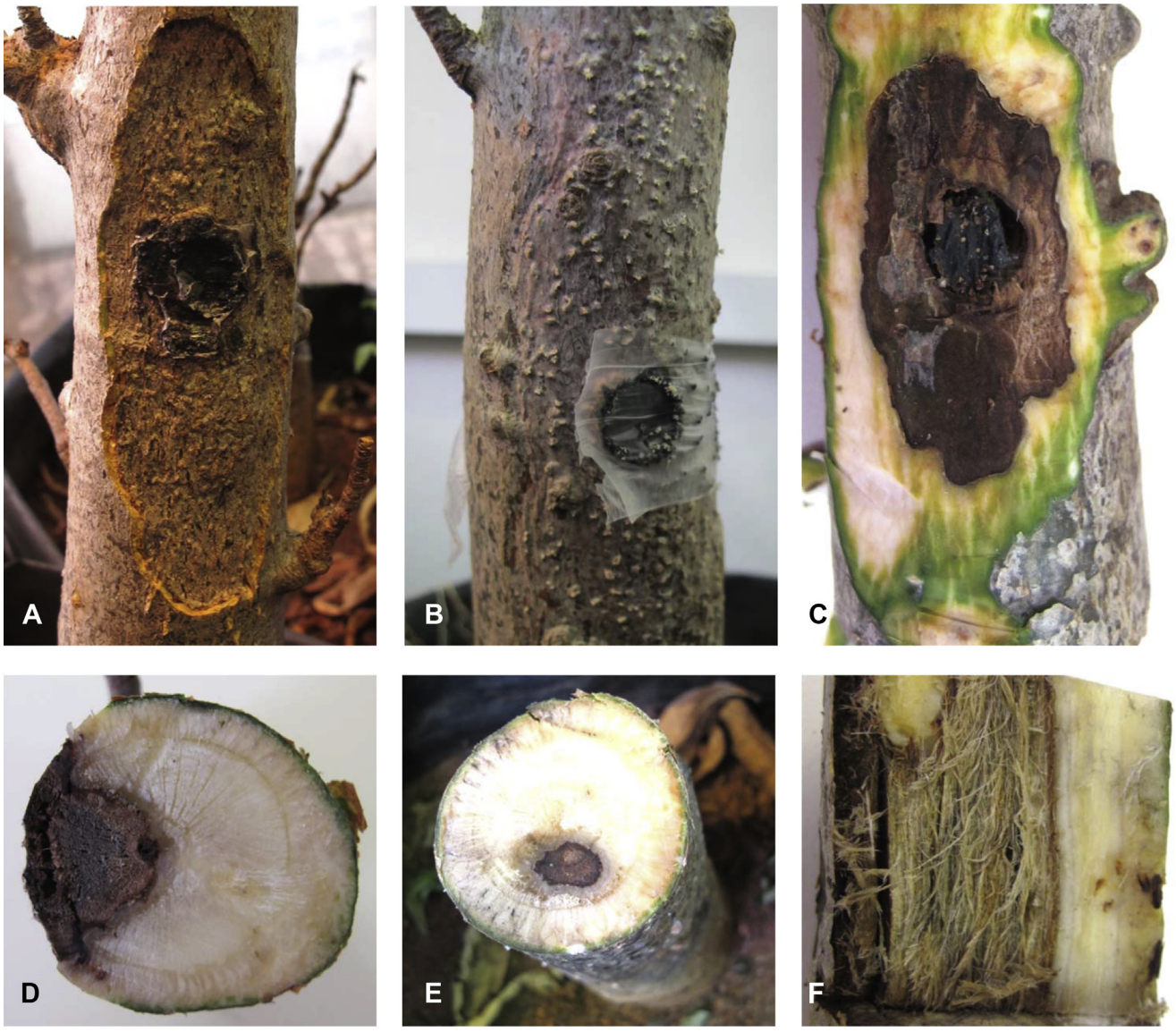

Fig 5 – (A) Sunken lesions around inoculation site, three weeks after inoculation with *Lasiodiplodia* isolate on baobab trees, (B) sporulation by *Lasiodiplodia* on bark and parafilm, (C) lesion under bark after six weeks, (D) lesion extending to middle of stem, (E) lesion inside stem extending past external lesion and (F) rotting symptom inside stem.

Mozambique was the only country where *L. gonubiensis* were found, bringing the total number of species from that country to four. These were from only seven isolates obtained from six trees. Two isolates of *L. pseudotheobromae* were collected from South Africa and Mozambique respectively.

Lasiodiplodia iraniensis was isolated from healthy and diseased trees in Benin, Cameroon, Senegal, South Africa, Madagascar and Mozambique. There was some sequence variation between the different isolates. A single isolate from Madagascar (CMW 35879) grouped closest to *L. thailandica*, but distinct from both *L. iraniensis* and *L. thailandica*.

A group of 30 isolates (Table 4), mostly from Cameroon, grouped incongruently between trees from the various loci and these isolates are, therefore, considered as hybrids. Most of the hybrids formed between *L. mahajangana* and *L. euphorbiicola*, while two isolates were hybrids with *L. exigua* and *L. euphorbiicola*. Four isolates from Cameroon formed hybrids between *L. iraniensis* and *L. euphorbiicola*, and/or *L. mahajangana*. There was one hybrid each from Senegal and South Africa, three from Benin and Zimbabwe each, and five from Madagascar. While there was only one isolate of *L. euphorbiicola* from Madagascar, all five of the hybrids from Madagascar were between *L. mahajangana* and *L. euphorbiicola*. This suggests that *L. euphorbiicola* is more prevalent in Madagascar than is apparent from this survey.

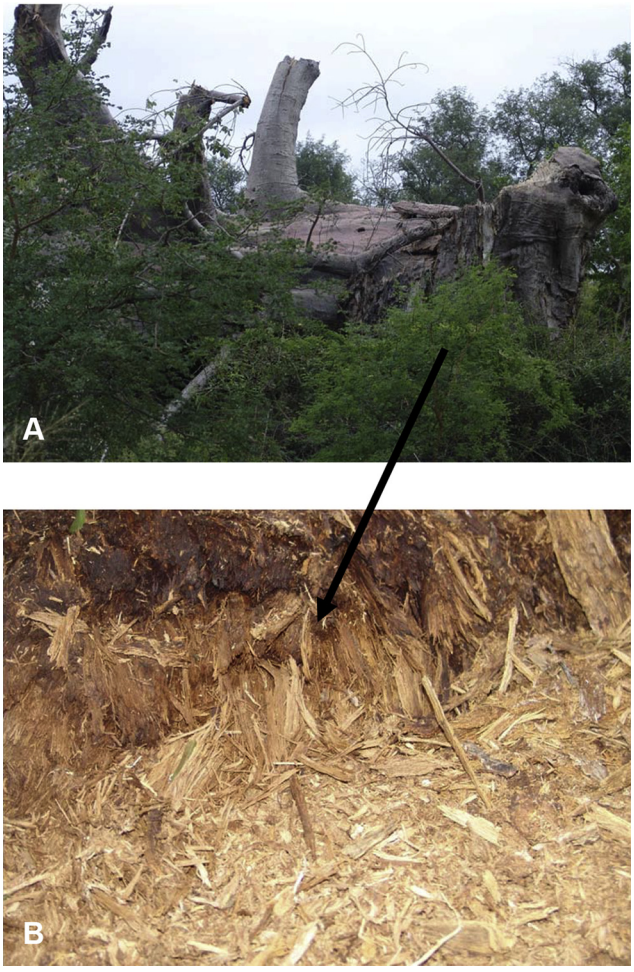

Fig 6 – Baobab tree in KNP that had recently collapsed (A) main stem broken due to rotten wood inside, (B) loose fibres of rotten wood inside main stem.

Pathogenicity trials

Sunken areas around the points of inoculation were observed on young baobab trees approximately three weeks after inoculation with the selected *Lasiodiplodia* species (Fig 5A). Some isolates sporulated profusely on the bark (Fig 5B) and parafilm, covering the inoculation sites. After six weeks, the lesions under the bark were measured (Fig 5C,D), but some lesions at the centres of the stems extended further up and down within the stem tissue than the lesions underneath the bark (Fig 5E). Some of the fungi caused severe rotting of the wood near the inoculation site (Fig 5F) and this resembled the wood rot observed in the trunks of recently fallen mature baobab trees (Fig 6).

Variation in the lesion lengths was observed associated with inoculations of different isolates of the same species (Fig 7). Both isolates of *Lasiodiplodia pseudotheobromae*, as well as the *Lasiodiplodia iraniensis* isolate, caused lesions that were significantly ($p < 0.001$) larger than those of the controls. Two of the three *Lasiodiplodia euphorbiicola* isolates tested caused significant lesions while the third isolate (CMW 33327) did not. Most of the *Lasiodiplodia mahajangana* isolates gave rise to only small lesions or did not result in lesion development. An exception was found with isolates CMW 36172 and CMW 36212 that were associated with lesions significantly larger than those of the controls.

Discussion

Phylogenetic inference based on four gene regions made it possible to identify numerous species of *Lasiodiplodia* occurring on baobabs. Importantly, the results show that several isolates of previously described *Lasiodiplodia* species, including the ex-type isolates of *Lasiodiplodia brasiliense*, *Lasiodiplodia laeliocattleyae*, *Lasiodiplodia missouriana*, and *Lasiodiplodia viticola*, as well as a group of isolates from baobab trees, grouped incongruently in trees derived from different loci. This incongruence could be explained only by hybridisation. The described species are, therefore, invalid and they have consequently been designated as the hybrid species *Lasiodiplodia ×brasiliense*, *Lasiodiplodia ×laeliocattleyae*, *Lasiodiplodia ×missouriana*, and *Lasiodiplodia ×viticola*. The isolates from baobab trees were identified as the hybrid species *L. ×brasiliense* and *L. ×laeliocattleyae*, together with *Lasiodiplodia crassispora*, *Lasiodiplodia euphorbiicola*, *Lasiodiplodia exigua*, *Lasiodiplodia gonubiensis*, *Lasiodiplodia iraniensis*, *Lasiodiplodia mahajangana*, *Lasiodiplodia pseudotheobromae*, and *Lasiodiplodia theobromae*.

The fact that evidence of hybridisation was found in ex-type as well as other isolates of described *Lasiodiplodia* species is not surprising. The broad host ranges and endophytic nature of these fungi facilitate their global movement with plant material. This brings related fungi that had speciated in allopatry into contact, which would be ideal for hybrids to form because these species are expected to not have evolved mating barriers in all cases (Brasier 1995; Brasier 2001). The large

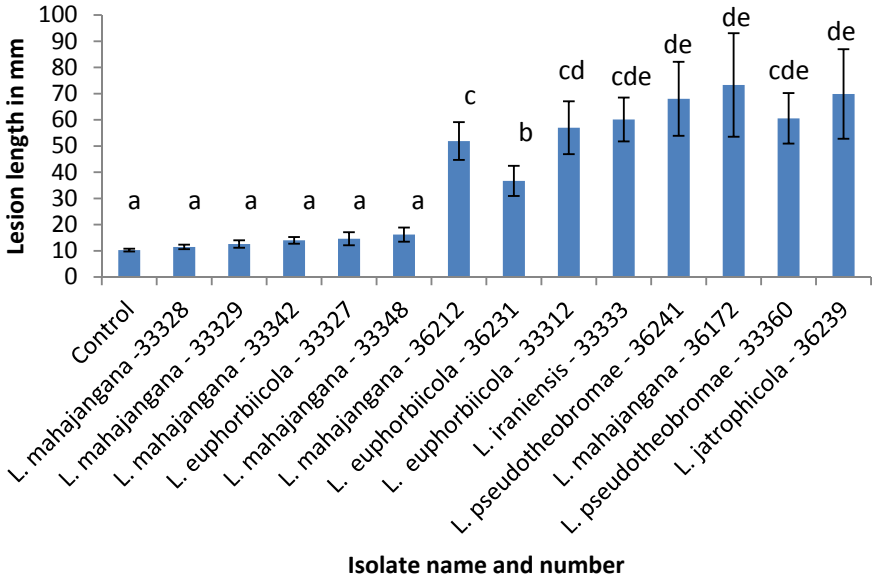

Fig 7 – Mean lesion length (mm) of pathogenicity trial with *Lasiodiplodia* isolates on young baobab (*Adansonia*) trees. Significant differences (p < 001) are indicated with different letters above the bars.

numbers of sexual and asexual spores produced by fungi also make successful hybridisation more likely because only a few of the millions of spores produced require a fitness advantage over the parental species. These fungi with new combinations of genes would then be able to outcompete the parental species or occupy a novel niche (Stukenbrock 2016).

The hybrid species and isolates identified in this study showed incongruence between different gene trees. This has also been found in the studies of endophytes of tall fescue grasses (Schardl & Craven 2003; Moon *et al.* 2004) and *Fusarium* (O'Donnell *et al.* 2000). The evidence that at least four of the previously described *Lasiodiplodia* species are hybrids, emphasises the importance of using multiple loci, and as many isolates as possible, to define cryptic species. In particular, interpretation of *tef*1-α data must be made with caution and not only in combination with ITS. This is because phylogenies based on the *tef*1-α locus commonly display incongruence with other gene trees. As part of this study and to facilitate future work, we have also presented a decision tree that can be used to identify other groups of hybrid fungi in the *Botryosphaeriaceae*.

Our study is not the first to observe hybrids in *Lasiodiplodia*, but is the first to describe these hybrid species. Sakalidis (2011) reported on *Lasiodiplodia* isolates that appeared to be hybrids of two different *Lasiodiplodia* species, where *Lasiodiplodia* hybrid 1 was similar to *L. pseudotheobromae* based on ITS and intermediate between *Lasiodiplodia parva* and *L. pseudotheobromae* based on *tef*1-α. Hybrid 2 was similar to *Lasiodiplodia citricola* based on ITS and intermediate between *L. parva* and *L. citricola* based on *tef*1-α. These species were, however, not described.

The 30 isolates considered as hybrids and collected from baobab trees in this study varied in the number of gene regions in which they grouped with different species. Some isolates grouped with *L. mahajangana* based on two loci and *L. euphorbiicola* based on the other two loci evaluated. Other isolates showed congruence based on three loci and they grouped with a different species based on only a single locus. It is, therefore, clear that hybrids can easily be overlooked when only one or two loci are used for identification, as has clearly occurred in many of the cases that we have described in this study. This appears to be a common problem in *Lasiodiplodia* and it is likely also true for other species in the *Botryosphaeriaceae*.

The hybrid isolates from baobab trees were classified based on information from four loci. Many hybrids have traditionally been classified based on morphology that was intermediate between that of the parental strains, or changes in pathogenicity (Newcombe *et al.* 2000; Joly *et al.* 2006). However, the similar morphology of *Lasiodiplodia* species and their broad host ranges would make it impossible to use morphology or pathogenicity for hybrid identification. A single locus has been used to infer hybridisation in diploid organisms or where a locus is duplicated (Nielsen & Yohalem 2001; Man in 't Veld *et al.* 2006; Man in 't Veld *et al.* 2012). It would appear that only single versions of the loci tested thus far are present in *Lasiodiplodia*, therefore hybrids cannot be detected in this way. The utilisation of multiple loci is currently the most efficient way to recognise hybrids in *Lasiodiplodia*.

Lasiodiplodia mahajangana was the species most often isolated from baobabs in Africa and it was isolated from healthy and diseased trees. Interestingly, this was also the species most commonly found on *Adansonia gregorii* in Australia where it caused lesions in a pathogenicity trial (Sakalidis *et al.* 2011a). The pathogenicity trials with the African isolates in the present study revealed considerable variation, with only two of the six isolates causing lesions. *Lasiodiplodia mahajangana* was isolated from all the countries sampled, and it appears to have a wide host range and worldwide distribution. This species was originally described from *Terminalia catappa*

in Madagascar (Begoude et al. 2010), but has subsequently been reported from various other hosts and countries. Some of the hosts and countries from which it has been collected include *A. gregorii*, *Santalum album*, *M. indica* and *Melaleuca* sp. in Australia (Sakalidis 2011); *Pistacia vera* in the USA (Inderbitzin et al. 2010) and *Euphorbia ingens* in South Africa (Van der Linde et al. 2011).

The second major group of isolates from baobabs clustered with *L. euphorbiicola*. Isolates of this species were obtained from seven of the nine countries where samples were collected, but not from South Africa and Mozambique. Pathogenicity trials revealed variability in aggressiveness, with one isolate not causing lesions, and two others used in the tests, causing significant lesions on baobab seedlings. *Lasiodiplodia euphorbiicola* was described from *Jatropha curcas* in Brazil (Machado et al. 2014) and is closely related to *L. parva*. *Lasiodiplodia euphorbiicola* and *L. parva* are identical based on four loci and differed only by six base pairs based on the *tef*1-α locus.

Species of *Lasiodiplodia* that were found in only one country included *L. gonubiensis* collected only in Mozambique, and *L. crassispora* isolated from diseased and healthy trees in Senegal. Although these species were found on baobab trees infrequently, they have been reported from different hosts in other countries and continents. For example, *L. crassispora* was described from *S. album* in Australia (Burgess et al. 2006). Consequently these species also have broad host and distribution ranges and may be present on baobab trees more often than is evident from this study.

Several isolates in the present study clustered with *L. iraniensis* and were found from Benin, Cameroon, Madagascar Senegal and South Africa. Most of the isolates were obtained from healthy trees, with the exceptions being those from Senegal and Benin. In a survey of fungi occurring on baobabs trees in Australia, Sakalidis et al. (2011a) found that *L. iraniensis* was the most aggressive species. The *L. iraniensis* isolate included in the current pathogenicity trial also gave rise to significant lesions. However, no *L. iraniensis* were isolated from the population of baobab trees with the highest incidence of disease observed in this study (Tsumkwe area in Namibia); only *L. mahajangana* and *L. euphorbiicola* were found from these trees. *Lasiodiplodia iraniensis* clearly has a worldwide distribution and wide host range having been described from *Mangifera indica* in Iran and reported on *Juglans* sp., *Citrus* sp. and *Salvadora persica* in the same country (Abdollahzadeh et al. 2010). *Lasiodiplodia iraniensis* has also been isolated from *A. gregorii* in Australia (Sakalidis et al. 2011a).

The worldwide occurrence of many of the *Lasiodiplodia* species in this study suggest that these species are being moved around the world. *Botryosphaeriaceae* occurring as endophytes in plants are efficient, opportunistic colonisers of plants (Slippers & Wingfield 2007) and *Lasiodiplodia* is probably being moved with plant material. Our discovery of four hybrid species and many hybrid isolates within *Lasiodiplodia*, raises concerns that introductions of new species may result in the formation of more hybrids. These hybrids can evolve more rapidly (Brasier 2001) and may be more aggressive or have wider host ranges than the parental species, as was found for both the poplar rust pathogen *Melampsora* ×*columbiana* (Newcombe et al. 2000) and *Verticillium longisporum*, a pathogen of crucifers (Inderbitzin et al. 2011). This emphasises an urgent need to restrict the global movement of plant material (Liebhold et al. 2012; Wingfield et al. 2015).

This study serves as a foundation towards understanding the distribution and role of endophytic *Lasiodiplodia* on baobabs in Africa. It is not clear whether these fungi play a role in the baobab deaths that have been observed. But the fact that some of the isolates tested caused substantial lesions and severe rotting of the stems, may be linked to the rotting of mature trees seen in the field. The global movement and distribution of these fungi deserves further study to fully understand the occurrence of different species in their countries of origin.

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